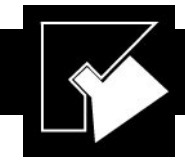
AFFINITY SEPARATION



K. Jones, Affinity Chromatography Ltd, Freeport, Ballsalla, Isle of Man, UK

Copyright © 2000 Academic Press

Introduction

Of the collection of separation technologies known as 'affinity', affinity chromatography is by far the most widely used variant. Affinity chromatography is becoming increasingly important as the speed of the revolution taking place in biotechnology processing increases. The concept of an 'affinity' separation results from a naturally occurring phenomenon existing within all biological macromolecules. Each biological macromolecule contains a unique set of intermolecular binding forces, existing throughout its internal and external structure. When alignment occurs between a specific site of these forces in one molecule with the site of a set of forces existing in another (different) molecule, an interaction can take place between them. This recognition is highly specific to the pair of molecules involved. The interactive mechanism can be converted into a universal mutual binding system, where one of the binding pair is attached to an inert matrix, packed into a column and used exclusively to capture the other matching molecule. When used in this (affinity) mode, the technique is probably the simplest of all chromatographic methods. It is, however, restricted almost exclusively to the separation and purification of biological macromolecules, and is unsuitable for small molecules.

Affinity chromatography or bioselective adsorption chromatography was first used in 1910, but it was only in the 1960s that affinity chromatography as practised today was developed as a purification technique. By the late 1970s the emergence of recombinant DNA technology for the manufacture of protein pharmaceuticals provided a new impetus for this highly specific chromatographic method, implemented by the demand for ever-increasing product purity implicit in regulatory frameworks devised by (amongst others) the USA's Food and Drug Administration (FDA). Finally, the need to reduce the cost of drugs is under constant scrutiny by many Governments, particularly those with controlled health schemes funded by revenue raised by taxation. These mutually incompatible pressures indicate the need for more efficient separation systems; the affinity technique provides the promise of meeting all necessary requirements.

Separation and purification methods for biological macromolecules vary from the very simple to the esoteric. The type of technique adopted is basically a function of source, the fragility of the molecule and the purity required. Traditionally, high purity protein pharmaceuticals have used multistage processing, but this is very inefficient as measured by the well-documented fact that 50–80% of total production costs are incurred at the separation/purification stage. In contrast, the highly selective indigenous properties of the affinity method offer the alternative of very elegant single-step purification strategies. The inherent simplicity and universality of the method has already generated a wide range of separation technologies, mostly based upon immobilized naturally occurring proteinaceous ligands. By comparing the 'old' technologies of 'natural' ligands or multistage processing with the 'new', exemplified by synthetic designed ligands, the most recent advances in affinity processing can be described.

Biological Recognition

As nature evolved, life forms had to develop a protective mechanism against invading microorganisms if they were to survive. Thus there is a constant battle between the cell's defence mechanism and the attacking microorganisms, a battle resolved by the cells generating antibodies (the immunoglobulins) able to recognize the protein coat of attacking microorganisms and signal killer cells to destroy the invaders before they cause harm to the host. Equally, if microorganisms were to survive, they had continually to mutate and change their protein coats to avoid detection by existing antibodies. The 'attack and destroy' process is a function of changes in the molecular structure in a specific part of the protein, with only the most minute of changes occurring at the surface of the protein. Evolution has thus designed a system where every protein has a very precise structure, but one which will always be recognized by another. One element of the interacting pair can be covalently bonded onto an inert matrix. The resulting chromatographic medium can then be packed into a column, and used to separate exclusively its matching partner from an impure mixture when added as a solution to the top of the column. This fact can be stated as follows – *for every protein separation problem there is always an affinity solution*. The process of producing a satisfactory medium is quite difficult.

DropBooks

The matching pair must be identified, and one of them isolated in a pure form. Covalent bonding onto an inert matrix in a stable manner must always allow the 'docking' surface of the protein to be positioned to make it available to the target protein. The whole also has to be achieved at an acceptable cost.

This technique has resulted in many successful applications, often using antibodies as the affinity medium (immunoaffinity chromatography), but large scale separations using these 'natural' ligands are largely restricted by cost and regulatory reasons. Although immunoaffinity chromatography is still widely practised, in recent years the evolution of design technologies has provided powerful new approaches to mimic protein structures, resulting in the development of synthetic ligands able to work in harsh operational environments and at low cost.

The Affinity Process

The affinity method is critically dependent upon the 'biological recognition' existing between species. By permanently bonding onto an inert matrix a molecule (the ligand) that specifically recognizes the molecule of interest, the target molecule (the ligate) can be separated. The technique can be applied to any biological entity capable of forming a dissociable complex with another species. The dissociation constant (K_d) for the interaction reflects the complementarity between ligand and ligate. The optimal range of K_d for affinity chromatography lies between 10^{-4} and 10^{-8} mol L^{-1}. Most biological ligands can be used for affinity purposes providing they can be immobilized, and once immobilized continue to interact successfully with their respective ligates. The ligand can be naturally occurring, an engineered macromolecule or a synthetic molecule. **Table 1** provides some examples of immobilized ligands used to purify classified proteins. The affinity method is not restricted to protein separations; nucleic acids and whole cells can also be separated.

The simplicity of the chromatographic process is shown in **Figure 1**. The ligand of interest, covalently bonded onto the inert matrix, is contained in the column, and a solution containing the target (the ligate) is passed through the bed. The ligand recognizes the ligate to the exclusion of all other molecules, with the unwanted materials passing through the column packing while the ligate is retained. Once the bed is saturated with the target molecule (as measured by the breakthrough point), contaminating species are washed through, followed by collection of the target molecule as a very pure fraction using an eluting buffer solution. Finally, the column is cleansed from any strongly adsorbed trace materials, usually by regeneration with a strong alkali or acid, making it available for many more repeat runs. An outstanding advantage of the affinity process is an ability to concentrate very dilute solutions while stabilizing the captured protein once adsorbed onto the column. Many of the in-demand proteins manufactured by genetically engineered microorganisms are labile, allowing only minute quantities to be present in the fermentation mix before they begin to deteriorate. An ability to capture these very small quantities while stabilizing them in the adsorbant phase results in maximization of yield, making massive savings in total production costs.

Although the technical processing advantages are clear there is a major difficulty in the application of affinity chromatography as understood by most practitioners today. Most ligands described in Table 1 suffer from two primary disadvantages: a lack of stability during use; and high cost. Fortunately these problems have now been overcome, and affinity chromatography is now accepted as the major separations technology for proteins.

Table 1 Affinity ligands and purified proteins

Immobilized ligand	*Purified protein*
Divalent and trivalent metal ion	Proteins with an abudance of his, tryp and cys residues
Lectins	Glycoproteins, cells
Carbohydrates	Lectins
Reactive dyes	Most proteins, particularly nucleotide-binding proteins
Nucleic acids	Exo and endonucleases, polymerases, other nucleic acid-binding proteins
Amino acids (e.g. lys, arg)	Proteases
Nucleotides, cofactors substrates and inhibitors	Enzymes
Proteins A and G	Immunoglobulins
Hormones, drugs	Receptors
Antibodies	Antigens
Antigens	Antibodies

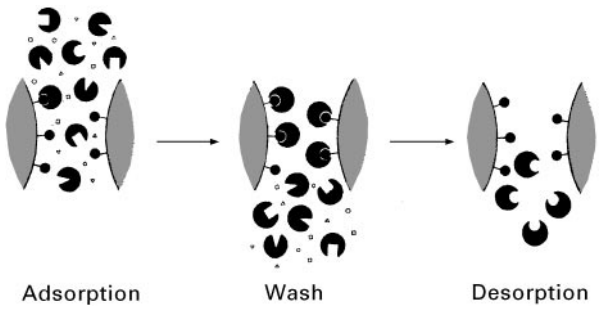


Figure 1 Schematic diagram of affinity chromatography.

Matrices

By definition matrices must be inert and play no part in the separation. In practice most play a (usually) negative role in the separation process. To minimize these disadvantages matrices have to be selected with great care. There is a theoretically perfect matrix, defined as consisting of monodispersed perfectly shaped spheres ranging from 5 to 500 μm in diameter, of high mechanical strength, zero nonspecific adsorption and with a range of selectable pore sizes from 10–500 nm, a very narrow pore size distribution and low cost. This idealized matrix would then provide the most efficient separation under all experimental conditions. As always, a compromise has to be reached, the usual approach being to accentuate the most attractive characteristics while minimizing the limitations, usually by manipulating the experimental conditions most likely to provide the optimum result.

The relative molecular masses of proteins vary from the low thousands to tens of millions, making pore size the most important single characteristic of the selected matrix. Very large molecules need very open and highly porous networks to allow rapid and easy penetration into the core of the particle. Structures of this type must therefore have very large pores, but this in turn indicates low surface areas per unit volume, suggesting relatively low numbers of surface groups to which ligands can be covalently attached. The matrix must also be biologically and chemically inert. A special characteristic demanded from biological macromolecular separations media is an ability to be sanitized on a routine basis without damage. This requires resistance to attack by cleansing reagents such as molar concentrations of strong alkali, acids and chaotropes. In contrast to analytical separations, where silica-based supports are inevitably used, silica cannot meet these requirements and is generally not favoured for protein separations. **Table 2** contains examples of support matrices used in affinity separations.

The beaded agaroses have captured over 85% of the total market for biological macromolecule separations, and are regarded as the industry standard to which all other supports are compared. They have achieved this position by providing many of the desirable characteristics needed, and are also relatively inexpensive. Beaded agaroses do have one severe limitation – poor mechanical stability. For analytical applications speed and sensitivity are essential, demanding mechanically strong, very small particles. Beaded agaroses are thus of limited use analytically, a gap filled by high performance liquid chromatography (HPLC) using silica matrices. For preparative and large scale operations other factors are more important than speed and sensitivity. For example, mass transfer between stationary phase and mobile phase is much less important when compared to the contribution from the chemical kinetics of the binding reaction between stationary phase and protein. Band spreading is also not a serious problem. When combined with the highly selective nature of the affinity mechanism, these factors favour the common use of large sized, low mechanical strength particles.

In recent years synthetic polymeric matrices have been marketed as alternatives. Although nonbiodegradable, physically and chemically stable, with good permeabilities up to molecular weights greater than 10^7 Da, the advantages provided are generally offset by other quite serious disadvantages, exemplified by high nonspecific adsorption. Inorganic matrices have also been used for large scale protein separations, notably reversed-phase silica for large scale recombinant human insulin manufacture (molecular weight approximately 6000 Da), but are generally not preferred for larger molecular weight products. A very slow adoption of synthetic matrices is

DropBooks

Table 2 Support matrices

Support matrix	*Operational pH range*
Agarose	2–14
Cellulose	1–14
Dextran	2–14
Silica	< 8
Glass	< 8
Polyacrylamides	3–10
Polyhydroxymethacrylates	2–12
Oxirane–acrylic copolymers	0–12
Styrene–divinylbenzene copolymers	1–13
Polyvinyl alcohols	1–14
N-Acryloyl-2-amino-2-hydroxy-1, 2-propane	1–11
PTFE	Unaffected

PTFE, polytetrafluoroethylene.

indicated as improvements are made to current materials and the prices of synthetics begin to approach those of agarose beads. Other factors resist any significant movement towards synthetic matrices. Most installed processing units are designed for low performance applications. Higher performance matrices would need reinstallation of new, much higher cost high performance plant; plant operators would need retraining; operating manuals would need rewriting; and plant and factory would need reregistration with the FDA. In combination, the implication is that penetration of high performance systems for large scale applications will be slow, and agarose beads will continue to dominate the market for protein separations.

Covalent Bonding

A basic requirement of all chromatographic media is the need for absolute stability under all operational conditions through many cycles of use. Consequently all ligands must be covalently bonded onto the matrix, and various chemistries are available to achieve this.

A number of factors are involved:

1. The performance of both ligand and matrix are not impaired as a result of the coupling process.
2. Most of the coupled ligand is easily accessible to the ligate.
3. Charged or hydrophobic groups are not generated on the matrix, so reducing nonspecific adsorption.
4. The immobilized ligand concentration is optimal for ligate bonding.
5. There is no leakage of immobilized ligand from the matrix.

Some ligands are intrinsically reactive (or can be designed to be so) and contain groups that can be coupled directly to the matrix, but most require coupling via a previously activated matrix. The affinity matrix selected must have an adequate number of appropriate surface groups onto which the ligand can be bonded. The most common surface group is hydroxyl. The majority of coupling methods involve the activation of this group by reacting with entities containing halogens, epoxy or carbonyl functional groups. These surface residues are then coupled to ligands through primary amines, hydroxyls or thiol groups, listed in **Table 3**.

Polysaccharides, represented by agarose, have a high density of surface hydroxyl groups. Tradition still dictates that this surface is activated by cyanogen bromide, but it is well established that this reagent forms pH-unstable iso-urea linkages, resulting in a poorly performing product. Furthermore CNBr-activated agarose needs harsh coupling conditions if high yields of final media are to be obtained, suggesting high wastage of often expensive ligands. This factor is particularly evident with fragile entities such as the very-expensive-to-produce antibodies, and yet many workers simply read previous literature and make no attempt to examine alternative far superior coupling methods. The advantages of mild coupling regimes are demonstrated in **Figure 2**, where the use of a triazine-activated agarose is compared to CNBr-activated agarose. Yield is significantly increased, largely by coupling under acidic rather than alkaline conditions.

Intermolecular Binding Forces

Almost all chromatographic separations rely upon the interaction of the target molecule with either a liquid phase or a covalently bonded molecule on the solid phase, the exceptions being those relying upon molecular size, e.g. molecular sieves and gel filtration. In affinity separations ligates are inevitably

Table 3 Activation materials

Activating reagent	*Bonding group on ligand*
Cyanogen bromide	Primary amines
Tresyl chloride	Primary amines, thiols
Tosyl chloride	Primary amines, thiols
Epichlorohydrin	Primary amines, hydroxyls, thiols
1,4-Butanediol diglycidyl ether	Primary amines, hydroxyls, thiols
1,1′-Carbonyldiimidazole	Primary amines, hydroxyls
Cyanuric chloride	Primary amines, hydroxyls
Divinylsulfone	Primary amines, hydroxyls
2-Fluro-1-methylpyriinium-toluene-4-sulfonate	Primary amines, thiols
Sodium periodate	Primary amines
Glutaraldehyde	Primary amines

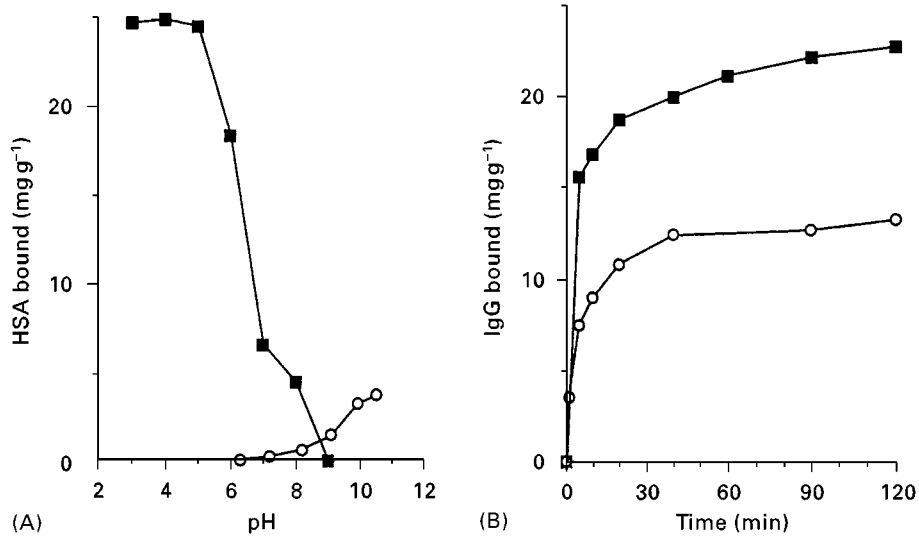


Figure 2 Triazine coupling. (A) Coupling of human serum albumin (HSA) to ready-acitivated supports as a funciton of pH. (B) Time course of coupling of human IgG to ready-activated supports at 4°C. ○, CNBr-activated agarose 4XL; ■, triazine-activated agarose 4XL.

complex biological macromolecules or assemblies, mostly or exclusively consisting of amino acids entities linked together in a specific manner. This complexity of structure provides many opportunities to exploit the physicochemical differences between the target molecule and the ligand to be used. Each structure contains the four basic intermolecular binding forces – electrostatic, hydrogen bonding, hydrophobic and van der Waals interactions – spread throughout the structure in an exactly defined spatial manner. The degree of accessibility and spatial presentation within the pore of the medium, and the strength of each force relative to each other, dictate whether these forces are utilized to effect the separation. The biological recognition between species is a reflection of the sum of the various molecular interactions existing between them, and this summation is fixed for the ligate. However, various ligands may be found that emulate some or all of the available binding forces to various degrees.

Affinity adsorbents are therefore assigned to one of three broad ligand categories: nonspecific, group specific or highly specific. Nonspecific ligands have only a superficial likeness to biological ligands and binding is usually effected by just one of the four binding processes described above. Although ion exchange materials can be used in a similar manner to affinity adsorbents, they only exhibit the single force of electrostatic binding. They are thus limited to relatively indiscriminate binding. In this case the only criterion for binding is that of an overall charge. Fortunately there are a vast number of biological ligands that can interact with more than one macromolecule and consequently group-specific ligands are commonplace. Since group-specific adsorbents retain a range of ligates with similar binding requirements, a single adsorbent may be used to purify a number of ligates. Group-specific ligates can be used when the desired ligate is present in high concentration, but this implies that some preprocessing has taken place and a concentration step interposed. The use of non/group-specific adsorbents can only offer partial separations. This results in having to apply several stages in series, each only capable of removing a proportion of the impurities. In contrast a highly selective ligand can exclusively remove the target in one step, but often the resulting complexes are very tightly bound, have low binding capacities, are easily denatured and are expensive to produce. Until recently these adsorbents were restricted to technically difficult isolations. Today the use of computer-assisted molecular modelling systems provides opportunities to investigate relationships between designed ligands and relevant protein structures. For the first time logical design approaches can be applied and consequently stable inexpensive ligands have now become available.

Analytical Scale-Up

Modern biotechnology uses two different types of chromatography. Analytical separations require that run time is minimized, while resolution and

DropBooks

sensitivity are maximized. In contrast, for preparative and process applications, the objective is to maximize purity, yield and economy. These techniques have developed separately, simply because biological macromolecules pose unique difficulties, making them unsuitable for 'standardized' analysis. A major influence on this division has been that scale-up usually occurs very much earlier in the development of a process, causing biochemists to turn to the traditional low performance methods of ion exchange (IE), hydrophobic interaction (HI) and gel permeation chromatography (GPC). The highly efficient affinity chromatography method was generally ignored, primarily because of the difficulty of having to develop a unique ligand for each separation rather than having 'off-the shelf' column packings immediately available from external suppliers. For analytical purposes high performance affinity liquid chromatography (HPALC) is a rarity, a function of the limited availability of suitable matrices (Table 2) and the affinity process itself.

Where quantifiable high speed chromatography is required, reversed-phase HPLC (RP-HPLC) has no equal. Unfortunately there is no general purpose method for biomolecules to parallel the inherent power of RP-HPLC. The success of RP-HPLC for analysis can be judged from the large number of published applications developed for the 'first wave' of protein pharmaceuticals manufactured by genetically engineered microorganisms. These extracellular (relatively) low molecular weight proteins include human insulin, human growth hormone and the interferons. However, as molecular size and fragility increase, so difficulties in using HPLC increase, a primary reason why much analysis is still conducted on low performance systems. Low performance systems are easily scaled up; RP-HPLC is not.

Biological separation systems must be aseptically clean throughout the process. The mixtures are inevitably complex and usually contain many contaminating similarly structured species. Such species can adsorb very strongly onto the medium, demanding post-use washing with very powerful reagents to sterilize and simultaneously clean the column. Silica-based matrices cannot survive this type of treatment, hence scale-up of analytical procedures is generally precluded. The first wave of commercial protein pharmaceuticals have generally proved to be relatively stable under high stress conditions. On the other hand intracellular proteins, often of high molecular weight, are unstable. Analysis by high performance RP-HPLC methods then becomes problematic. Demand for fast, high resolution, analytical methods will continue to increase for on-line monitoring and process validation. Such techniques have already been used to determine degradation of the target protein (for example deamidated and oxidized elements); to identify previously unidentified components; to establish the chromatographic identity between recombinant and natural materials; to develop orthogonal methods for the identification of unresolved impurities; and for many other demanding analytical approaches.

Affinity versus Traditional Media

When projects are transferred from research to development two sets of chromatographic techniques are carried forward: analysis, usually based on RP-HPLC; and larger scale serialized separation steps, often incorporating traditional methods of ion exchange, hydrophobic interaction and gel permeation chromatography. Major decisions have to be taken at this juncture – to scale up the separation processes developed during the research phase or to investigate alternatives. Regulatory demand and shortened patent lifetimes compel managements to 'fast track' new products. Commercial pressure is at a maximum. Being first to market has the highest priority in terms of technical and commercial reward. Very little time is left to explore other separation strategies. It is known that serial application of IE, HI and GPC inevitably leads to very high manufacturing costs, but which comes first? Most often the decision is taken to begin manufacture using unoptimized separations as defined in research reports. It is only in retrospect that very high production costs become apparent. By then it is too late – regulatory systems are firmly in place.

There is an alternative. If researchers were more aware of process economics and the consequences of regulatory demand, selection of superior separation processes could then result. Although most researchers are fully aware of the advantages of single-step affinity methods, paradoxically the high selectivity advantage of affinity chromatography is also a weakness. Suitable off-the-shelf affinity adsorbents are often unavailable, in which case an adsorbent has to be custom synthesized. Since the majority of biochemists have no desire (or time) to undertake elaborate chemical synthesis, antibody-based adsorbents are commonly used. However, raising suitable antibodies and purifying them before immobilization onto a preactivated support matrix is an extremely laborious procedure. In addition, proteins are so often tightly bound to the antibody that subsequent elution involves some degree of denaturation and/or loss of acitivity. Ideal media require the incorporation of elements of both nonselective and selective adsorbents to provide adsorbents with a general applicabil-

ity. If stable, highly selective and inexpensive affinity ligands were available, then opportunities would exist for researchers to develop efficient high yield separations even in the earliest phases of investigation. These systems could then be passed forward to production with the knowledge that optimally efficient separations are immediately achievable.

Production costs of any pure material reflect the absolute purity level required and the difficulty of achieving it. Therapeutic proteins have high purity requirements and the larger the administered dose, the purer it has to be. Since many protein pharmaceuticals will be used at high dose levels, purities need to exceed 99%, occasionally up to 99.999%. That these purities can be met by traditional methods is possible, but it is widely documented that the application of such methods massively increases production costs. Between 50 and 80% of total production costs of therapeutic proteins are incurred at the purification stage. The manufacturing cost of a product is directly related to its concentration in the mother liquor; the more dilute it is, the higher the cost of recovery. Since traditional purification processes on their own cannot selectively concentrate a target protein to the exclusion of all others, they have to be used in series. The number of stages required can vary between four and 15. Each step represents a yield loss, and incurs a processing cost. Yields of less than 20% are not uncommon. **Figure 3** shows an enzyme purified in multiple stages and by a one-step affinity process.

It was these limitations that caused biochemists to examine highly selective ligands. Almost any compound can be used as an affinity ligand provided it can be chemically bonded onto a support matrix and, once immobilized, it retains its ability to interact with the protein to be purified. The ligand can be a simple synthesized entity or a high molecular weight protein. The affinity technique is theoretically of universal application and any protein can be separated whatever its structure and origin. As always, there are major limitations. The most effective affinity ligands are other proteins. Unfortunately such proteins are difficult to find, identify, isolate and purify. This results in high costs. An even greater deterrent is that most proteins are chemically, catalytically and enzymically unstable, a particularly unattractive feature if they are to be used for the manufacture of therapeutic substances; and regulatory authorities generally reject applications using proteinaceous ligands.

In anticipating that one day stable inexpensive affinity media would be in demand, a team led by C.R. Lowe began an investigation into which synthetic ligand structures offered the greatest possibility of developing inexpensive stable ligands. It was concluded that structures that could be manipulated into specific spatial geometries and to which intermolecular binding forces could easily be added offered the highest chance of success. Model compounds were already available; the textile dyes.

Synthetic Ligands

Textile dyes had already proved to be suitable ligands for protein separations. Blood proteins, dehydrogenases, kinases, oxidases, proteases, nucleases, transferases and ligases can be purified by a wide variety of dyes. However, they did not prove to be the breakthrough so eagerly awaited. An essential feature of all chromatographic processes is exact repeatability from column to column, year after year. Textile dyes are bulk chemicals, most of which contain many by-products, co-produced at every stage of the dye

DropBooks

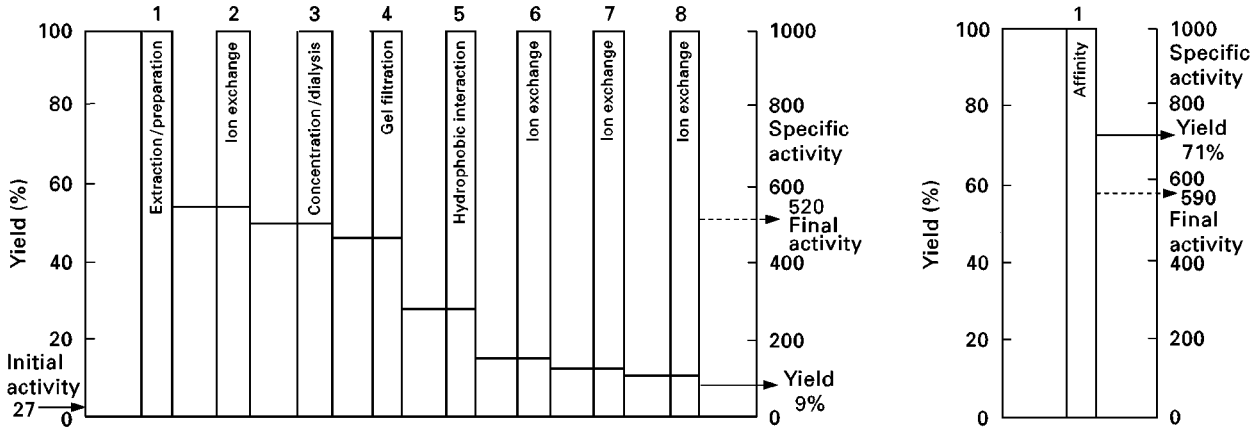


Figure 3 Comparison of multistep versus affinity separation.

manufacturing process. This fact alone makes reproducibility problematic. Furthermore, the bonding process between dye and matrix was poorly researched. This resulted in extensive leakage. All commercially available textile dye products leak extensively, especially under depyrogenating conditions (**Figure 4**). Despite these limitations, it was recognized that dye-like structures had a powerful underlying ability to separate a very diverse range of proteins. Their relatively complex chemical structures allow spatial manipulation of their basic skeletons into an infinite variety of shapes and configurations. Proteins are complex three-dimensional (3-D) structures and folds are present throughout all protein structures. An effective ligand needs to be shaped in such a manner that it allows deep insertion into a suitable surface fissure existing within the 3-D structure (**Figure 5**). In contrast, if the ligand only interacts with groups existing on external surfaces, then nonspecific binding results and proteins other than the target are also adsorbed. A much more selective approach is to attempt to insert a ligand into an appropriate fold of the protein, and add binding groups to correspond with those present in a fold of the protein. If all four of the basic intermolecular forces (Figure 5: W, electrostatic; X, hydrogen bonding; Y, van der Waals; Z, hydrophobic) align with the binding areas in the protein fold, idealized affinity reagents result. The use of spacer arms minimizes steric hindrance between the carrying matrix and protein.

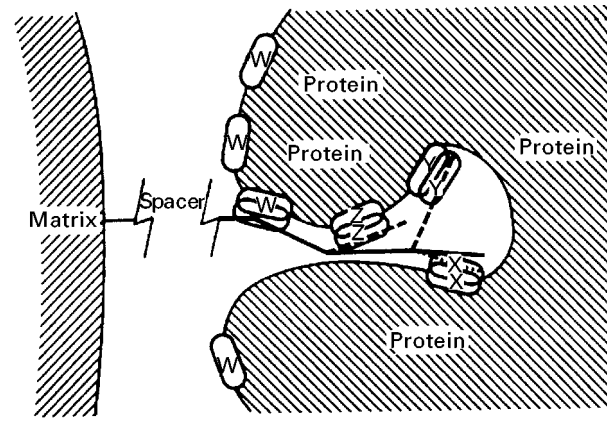


Figure 5 Schematic representtion of ligand–protein interaction. W, electrostatic interaction; X, hydrogen bonding; Y, van der Waals interaction; Z, hydrophobic interaction. ———, original backbone; - - -, new structure added; ···, original backbone move; ⬭, fields of interaction.

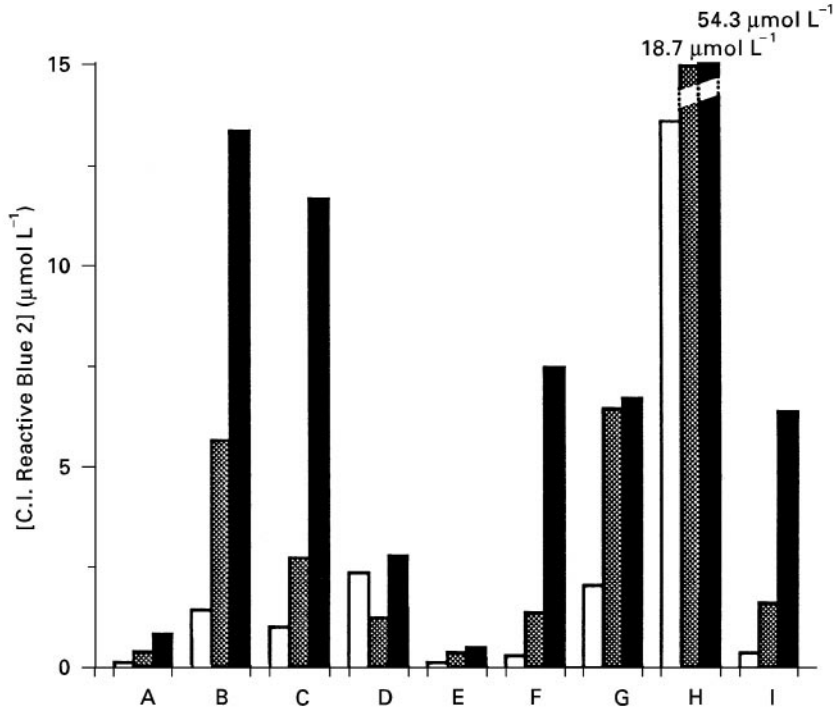


Figure 4 Leakage of blue dye from various commercial products. □, 0.1 mol L^{-1} NaOH; ▩, 0.25 mol L^{-1} NaOH; ■, 1 mol L^{-1} NaOH. Key: A, Mimetic Blue 1 A6XL (affinity chromatography); B, Affi-Gel Blue (Bio-Rad); C, Blue Trisacryl-M (IBF); D, Fractogel TSK AF-Blue (Merck); E, C.I. Reactive Blue 2 polyvinyl alcohol-coated perfluropolymer support; F, Blue Sepharose CL-6B (Pharmacia); G, immobilized Cibacron Blue F3G-A (Pierce); H, Cibacron Blue F3G-A = Si500 (Serva); I, Reactive Blue 2-Sepharose CL-6B (Sigma).

The final step is to design appropriate bonding technologies to minimize potential leakage. Until recently this type of modelling was a purely theoretical exercise. It was only the introduction of computer-assisted molecular modelling techniques that allowed the theory to be tested. Before the arrival of logical modellling the discovery of selective ligands was entirely based upon empirical observation, later followed by a combination of observation, experience and limited assistance from early computer generated models. Although several novel structures evolved during this period, a general approach to the design of new structures remained elusive. At this time only very few 3-D protein structures were available, again greatly restricting application of rational design approaches. As more sophisticated programmes, simulation techniques, protein fragment data and many more protein structures were released, logical design methods were revolutionized. However, many millions of proteins are involved in life processes, and it is clear that many years will elapse before the majority of these will be fully described by accurate models. Consequently intuition and experience will continue to play a major role in the design of suitable ligands. Of available rationally designed synthetic molecules, the Mimetic™ range can currently separate over 50% of a randomly selected range of proteins. Stability under depyrogenating conditions has been demonstrated for these products (**Figure 6**). This results in minimal contamination from ligand and matrix impurities, substantial increases in column lifetime, and improvements in batch-to-batch reproducibility.

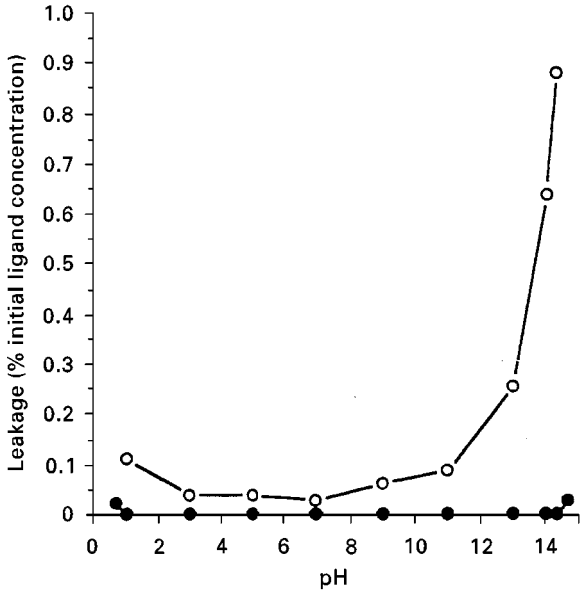


Figure 6 Comparison of ligand leakage from mimetic ligand affinity adsorbent A6XL (●) and conventional textile dye agarose (○).

Rational Design of Affinity Ligands

Modification of Existing Structures

The first example of a rational design of new biomimetic dyes used the interaction between horse liver alcohol dehydrogenase (ADH) and analogues of the textile dye Cibacron Blue F3G-A (**Figure 7**). It had been established that the parent dye binds in the NAD^+-binding site of the enzyme, with the anthraquinone, diaminobenzene sulfonate and triazine rings (rings A, B and C, respectively, in Figure 7) apparently adopting similar positions to those of the adenine adenosine ribose and pyrophosphate groups of NAD^-. The anthraquinone ring (A) binds in a wide apolar fold that constitutes, at one end, the adenine bridging site, while the bridging ring (B) is positioned such that its sulfonate group interacts with the guanidinium side chain of Arg271 (**Figure 8**). Ring C binds close to where the pyrophosphate bridge of the coenzyme binds with the reactive triazinyl chlorine adjacent to the nicotinamide ribose-binding site. The terminal ring (D) appears to be bound in a fold between the catalytic and coenzyme binding domains, with a possible interaction of the sulfonate with the side chain of Arg369. The binding of dye to horse liver ADH resembles ADP binding but differs significantly at the nicotinamide end of the molecule with the mid-point position of ring D displaced from the mid-point position of the nicotinamide ring of NAD^+ by about 1 nm. Consequently a number of terminal-ring analogues of the dye were synthesized and characterized in an attempt to improve the specificity of dye binding to the enzyme. **Table 4** lists some of the analogues made by substituting –R in the D ring (Figure 7), together with their dissociation constants. These data show that small substituents bind more tightly than bulkier groups, especially if substituted in the *o*- or *m*-positions with a neutral or anionic group. Further inspection of the computer model given as Figure 8 showed that the dye analogues were too short and rigid to bind to horse liver ADH in an identical manner to the natural coenzyme, NAD^+. Consequently analogues of the parent dye were designed and synthesized with central spacer functionalities to increase the length and flexibility of the molecule (**Figure 9**). This product proved to be some 10 times superior to any previously synthesized compound. This work provided the first proof that rationally designed molecules could be converted into stable, inexpensive, chromatographic media, while providing the most remarkable separations.

DropBooks

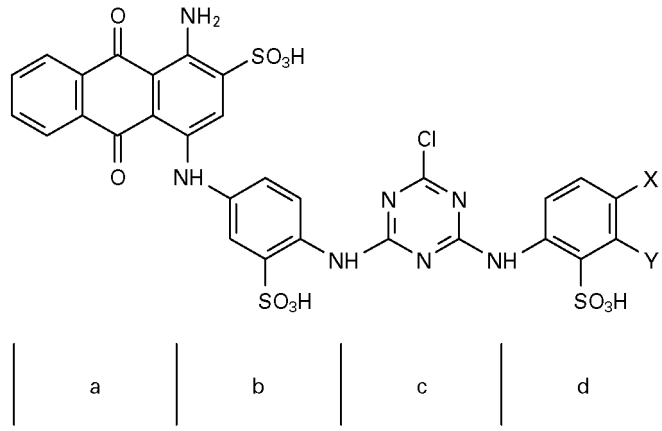


Figure 7 Principal structural elements of the anthraquinone dye, Cibacron Blue F3G-A.

De Novo Design

Most early efforts in proving the rational design technology was based upon dye structures. To date all dyes considered have been anionic, presumably because the charged chromophores of these ligands mimic the binding of naturally occurring anionic heterocycles such as NAD^+, $NAPD^+$, ATP, coenzyme A, folate, pyridoxal phosphate, oligonucleotides and polynucleotides. However, some proteins, particularly proteolytic enzymes, interact with cationic substrates. The trypsin-like family of enzymes forms

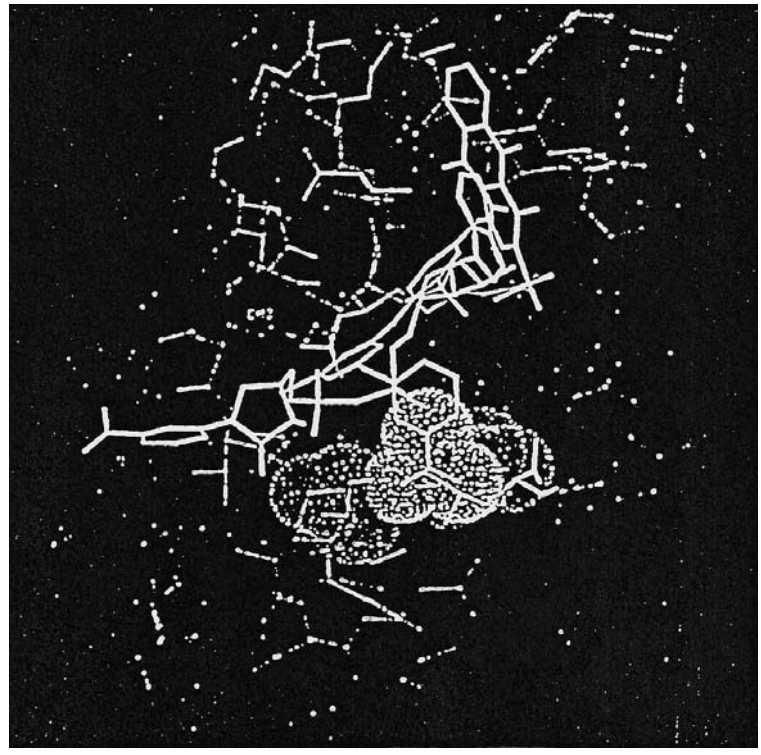
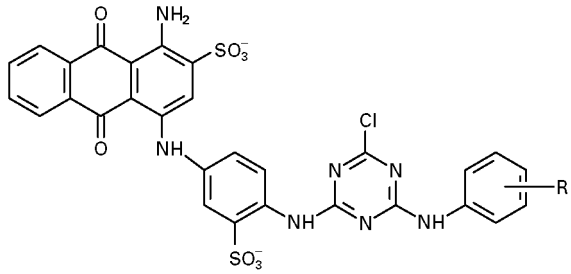


Figure 8 Putative binding pocket for the terminal-ring analogue (*m*-COO^-) of Cibacron Blue F3G-A in the coenzyme binding site of horse liver alcohol dehydrogenase (ADH). The site lies lateral to the main coenzyme binding site and comprises the side chains of two juxtaposed cationic residues Arg47 and His51.

Table 4 Apparent affinities of terminal-ring analogues of anthraquinone dyes for horse liver alcohol dehydrogenase (ADH)



R	*Apparent* K_d (μmol L^{-1})
m-COO^-	0.06
H	0.2
o-COO^-	0.2
o-SO_3^-	0.4
m-SO_3^-	1.6
m-CH_2OH	4.5
m-$CONH_2$	5.7
p-COO^-	5.9
p-SO_3^-	9.3
p-PO_3^{2-}	10.5
p-$N^+(CH_3)_3$	172.0

one of the largest groups of enzymes requiring cationic substrates and includes enzymes involved in digestion (trypsin); blood clotting (kallikrein, thrombin, Factor Xa); fibrinolysis (urokinase, tissue plasminogen activator) and complement fixation. These enzymes possess similar catalytic mechanisms and bind the side chains of lysine or arginine in a primary pocket proximal to the reactive serine (Ser195), with specificity being determined partly by the side chain of Asp189 lying at the bottom of the pocket, and partly by the ability of the individual enzymes to form secondary interactions with the side chains of other nearnearby substrate amino acids. For example, tissue kallikrein differs from pancreatic trypsin in that it displays a marked preference for phenylalanine in the secondary site, probably because the phenyl ring on the phenylalanine residue neatly slips into a hydrophobic wedge-shaped pocket between the aromatic side chains of residues Trp215 and Tyr99 (**Figure 10**). Specificity for the secondary amino acid residue is less stringent in trypsin since Tyr99 is replaced by Ala99 and the hydrophobic pocket cannot be formed. By designing a mimic for the Ph–Arg dipeptide should result in a specificity for kallikrein. **Figure 11** uses *p*-aminobenzamidine and phenethylamine functions substituted on a monochlorotriazine moiety. However, the active site of pancreatic kallikrein lies in a depression in the surface of the enzyme. The expected steric hindrance is eliminated by insertion of a hexamethylene spacer arm between the designed ligand and the matrix. After synthesis of this medium it was demonstrated that purified pancreatic kallikrein was strongly bound, with over 90% of activity being recovered on elution with 4-aminobenzamidine, whereas trypsin appeared largely in the void of the column. This medium was able to purify kallikrein 110-fold from a crude pancreatic acetone powder in a single step.

There is an alternative to the rational design approach – the use of combinatorial libraries.

Combinatorial Libraries

The driving force behind the development of combinatorial libraries has been the many failed attempts to design therapeutic substances using theoretical knowledge allied to rational design; very few such approaches succeeded. In contrast, combinatorial library design is now thought by some to provide the best opportunity of discovering new novel peptides and small molecule structures for pharmaceutical application. A quite natural extension of the concept is to use combinatorial libraries to discover ligands capable of achieving highly efficient protein separations. When directed at drug discovery the earliest workers built libraries from peptides. For ligands libraries will generally utilize simple chemical molecules and occasionally smaller peptides. To distinguish this subsection from the earlier methods a convenient designation is the term Chemical Combinatorial Library (CCL)™.

Although procedures for rationally designed ligands are well established, the newness of CCL suggests that CCL design of affinity ligands should be regarded as embryonic rather than immediately available for commercial application. There are thus two diametrically opposed systems – the rational design process, based on logic, experience and knowledge; and CCL which is illogical and completely random. One description of CCL is 'a method of increasing the size of the haystack in which to find your needle'. A very recent approach is to combine both rational and CCL techniques, a process termed 'intelligent' combinational design. At the time of writing there are no published examples of ligands derived from CCL, although patents have been filed in this area.

Regulations and Drug Master Files

For researchers the relevance of regulations often seems remote, and yet the decisions taken in even the earliest stages of research can take on a great significance if the target product becomes a commercial

DropBooks

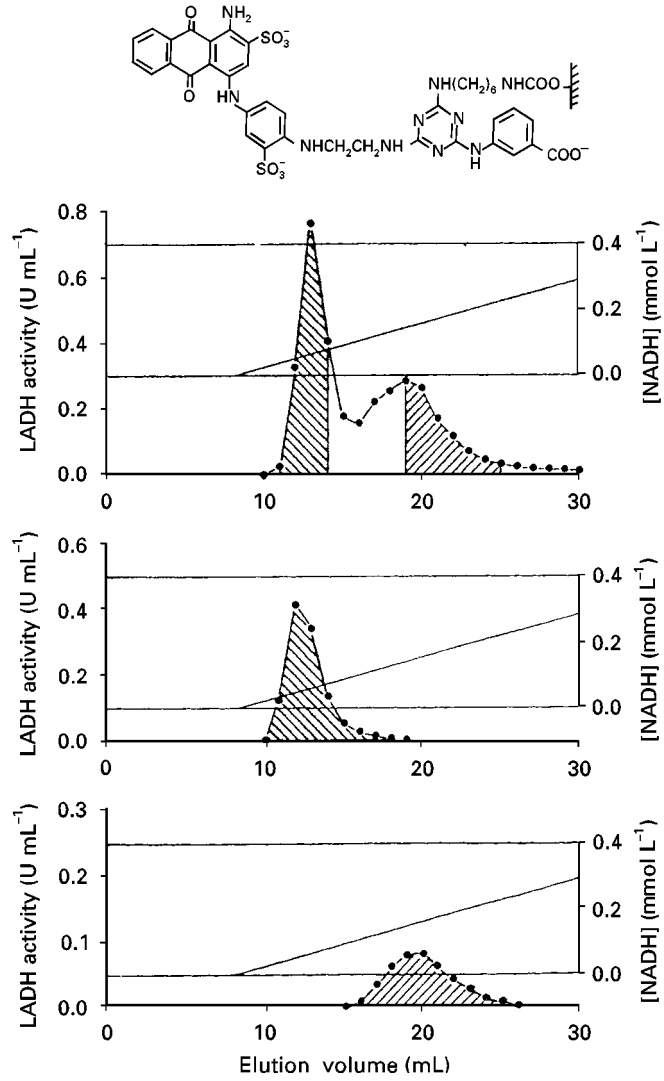


Figure 9 Horse liver alcohol dehydrogenase separation. Using the modified Cibacron Blue F3G-A (Figure 7) structure given above, the selectivity is greatly enhanced making it possible to separate the isoenzymes.

reality. It is regrettable that many researchers slavishly follow previously published data on a given separation problem without giving thought to the longer-term implications of their decisions. Sections above describe the adverse economic effects of multi-stage processing, but an equally important factor is regulatory issues. The most widely used regulations are those defined by the USA's Food and Drug Administration (FDA). Any company wishing to import products relevant to regulations existing in the USA must conform exactly to FDA requirements; drugs in particular are very strictly controlled. Detailed descriptions of any plant and process used in drug manufacture have to be lodged in documented form with the FDA, wherein every aspect of process description is given. This must include raw material definition and suppliers, stability data for every step of the process, formulation methods, packaging, labelling, toxicity data and so on. The documentation has to be revised annually and any changes notified. Furthermore plant and process is open to inspection at all times for full audit of procedure. There is one large anomaly within the regulations. The largest volume of material in contact with a drug during manufacture is water, solvents and salts, all of which are exactly defined in terms of their physicochemical characteristics. The next largest is chromatographic media; ambiguously media do not have to be described in the same detail.

The outstanding stability of the synthetic chromatography media provides an excellent opportunity to develop and register Drug Master Files

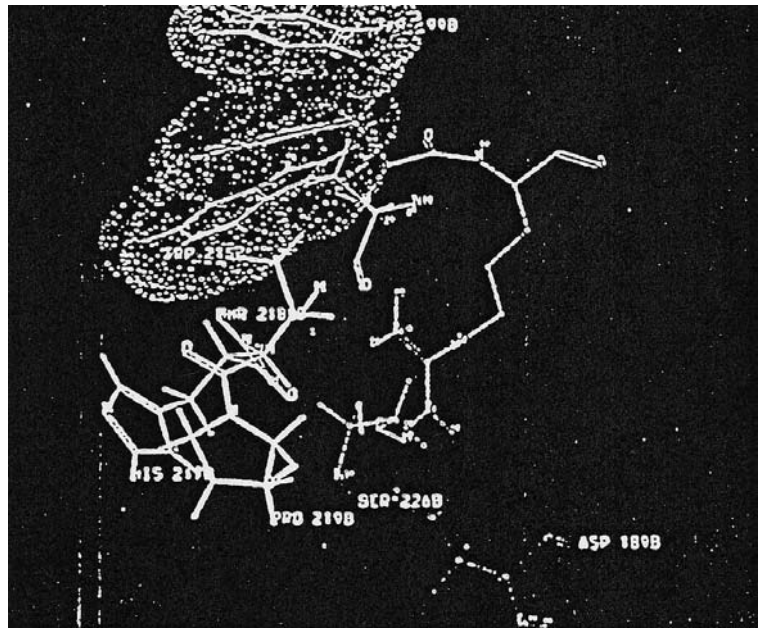


Figure 10 Model of the Phe–Arg dipeptidyl substrate bound in the acitve site of porcine pancreatic kallikrein. The illustration shows Asp189 at the bottom of the primary binding pocket as well as the side chains of Tyr99 and Try215, which form the secondary binding pocket, with the phenyl ring of the Phe residue sandwiched between the hydrophobic side chains of these residues.

DropBooks

(DMFs) with the FDA. DMFs allows companies to use synthesized ligands for very high purity protein pharmaceuticals with total confidence. New Drug Applications (NDA) and Investigational New Drugs (IND) documents incorporating such stable affinity media can now be submitted to the FDA, safe in the knowledge that all appropriate information is on file. The effective guarantee of minimum quality standards and Good Manufacturing Practice (GMP) is an integral part of a DMF. Few researchers selecting a specific medium consider the long-term implications of stability under depyrogenating conditions, the number of cycles that can be achieved (lifetime in use), its availability in bulk, whether it is manufactured under aseptic conditions and the price when supplied in bulk. If the researcher makes a good initial selection, the research data produced can be utilized in development phases with confidence. 'Fast tracking' is facilitated, with minimum aggravation, maximum efficiency and minimum purification costs.

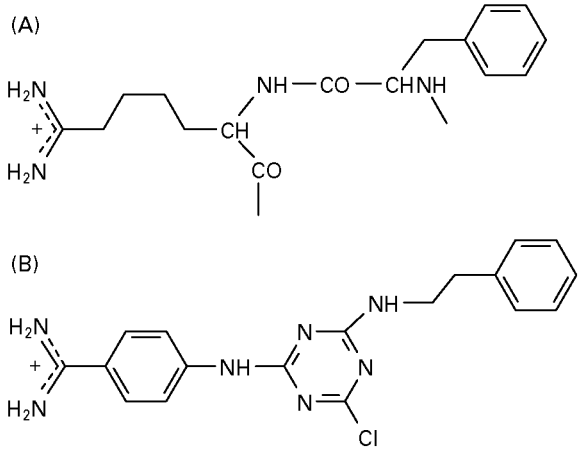


Figure 11 Comparison of the structures of (A) the Phe–Arg dipeptide, and (B) the 'biomimetic' ligand designed to bind at the active site of the porcine pancreatic kallikrein.

Alternative Affinity Approaches

Aqueous two-phase systems have been extensively applied to bimolecular purifications, by attaching affinity ligands to one of a pair of phase-forming polymers, a method known as *affinity partitioning*. Although a substantial body of research literature is available, few systems appear to have been adopted for commercial purposes. Reactive dyes, with their simple and well-defined coupling chemistries, have generally been favoured as the active ligand. The advantage of affinity partitioning is that the process is less diffusion-controlled, binding capacities

are high and the recovery of bound proteins is easier, created by the process operating with fewer theoretical plates than those generated by chromatography columns. This technique has also been combined with *affinity precipitation*, where a homobifunctional ligand composed of two ligand entities connected by a spacer (for example a bis-dye) is used. However, even in combination this approach suffers from considerable nonspecific binding and relatively low purification factors. A review of this combination suggests that it is more suited to large scale, low purity products. In contrast, perfluorocarbon emulsion chemistry utilizing mixer-settlers may offer more promise. By using a series of mixer-settlers connected in a loop a continuous process has been developed. A ligand (usually a reactive dye) is covalently bonded to a high density perfluorocarbon emulsion and contacted with the crude protein solution. After settling in the first tank the emulsion is pumped to a second settler and washed before passing to the third settler for elution. The emulsion is regenerated in the fourth settler. The supernatants from each settler, still containing some unbound target protein, are normally discarded. Although reasonable recoveries and yields are obtained, significant development is needed for this type of system to become competitive with conventional chromatography column methods.

Another favoured research approach to improving efficiency is to use *expanded beds*. Various techniques have been tried, with the primary objective of eliminating the 'solid bed' effect, where the bed acts as a filter, trapping insolubles and creating significant back-pressure. By partially removing the normal constraints of upper and lower retaining frits, which pack the particles tightly in the bed, the particles can expand, thereby releasing trapped solid impurities. Consequently longer operational cycles and higher flows result. One limitation of the expanded bed system is that adsorption can only be carried out in one stage, resulting in a less efficient process.

Expanded beds are only an intermediate stage towards *fluidized beds*. Several variations of fluidized bed technology have been adopted to evaluate them for affinity processing. One example is the use of perfluorocarbon emulsions in a countercurrent contactor. The affinity perfluorocarbon emulsion is loaded with crude source material into the base of a column in a similar manner to that of an expanded bed. The adsorbent is then removed from the base of the bed and carried forward through four identical contactors where washing, elution and regeneration are carried out successively. This process is claimed to improve significantly removal of target proteins compared to an expanded bed system.

Affinity Membranes

Ultrafiltration membranes are commonly employed as a 'polishing' stage of multistage separation processes for several commercially important proteins. Consequently attaching standard affinity ligands to create affinity membranes has become an actively researched area. The most obvious advantage of a membrane structure is the high rate of transport of the medium through the porous structure by filtration, thus minimizing the normally encountered diffusion limitations of mass transfer. High adsorption rates are achieved, especially if long distance electrostatic interactions are involved in the binding mechanism. However, in contrast to ion exchange membranes, similar high transport effects are not observed when used in the affinity mode, eliminating much of the initial attraction of this form of device. Other theoretically attractive features included: an inherent ability to control pore size across a very wide range, offering an opportunity to increase capacity of a given system; and ability to operate in either batch mode or filtration mode. In both cases experimental data have not confirmed these assumed advantages; a 10-fold change in the pore size resulted in only a two-fold capacity increase and when in filtration mode, although adsorption is fast, severe peak broadening on elution is experienced.

The chemistry relevant to particulate media is identical to that required for membranes, in effect making the systems compatible and allowing an easy technology interchange. The covalent bonding of affinity ligands to the surface of a membrane follows exactly the same chemistry as that applied to particulate media, and the same adsorption/desorption principles apply to both. Consequently the only difference between membrane systems and those of conventional chromatography is the exploitation of the characteristics of the membrane matrix compared with a particulate bed. Although the high mechanical strength of membranes is one major advantage, plus the scale-up is claimed to be very easy by stacking membranes (although scaling affinity columns is also very straightforward), it has been discovered that if the pressure drop across the membrane is too high sealing problems occur; the mobile phase then flows beyond the edges and past the membranes. Furthermore affinity membranes should be capable of use with unclarified extracts, but is has been generally observed that membrane capacity and lifetime are progressively reduced with time of use. Even with clarified broths, membrane fouling regularly occurs. This is almost certainly the reason why affinity membranes have not found favour in large scale processing.

Conclusion

Protein separations can be achieved by a variety of affinity techniques, but separations in the chromatography mode are by far the most widely used. Nature defined an appropriate pathway to highly efficient separation – utilization of the phenomenon of the automatic recognition mechanism existing between a given protein and at least one other. By covalently bonding one of the pair onto an inert matrix a theoretically simple separation process can be devised. Although these immunoaffinity separations are widely practised today, severe limitations exist, not least of which are cost and instability of the affinity medium when in use. As modern design aids have become commonplace, in conjunction with newer techniques such as the development of combinatorial library arrays, it has proved possible to mimic nature and replace immunoaffinity matrices by specifically designed synthetic ligands. These new ligands not only accurately emulate the exquisite precision of the natural protein–protein interaction mechanisms, but also provide the opportunity to manipulate the ligand structures, thus offering far more efficient separations than any previously achieved. For a given protein, from whatever source and at any dilution, it is now possible virtually to guarantee that a highly cost-effective and highly efficient separation process can be developed for eventual commercial use.

Designed ligand processes have already been adopted for several very large biotechnology projects scheduled to manufacture bulk protein pharmaceuticals. A mandatory part of any new protein pharmaceutical process is the acceptance by regulatory authorities of the separation process involved. That synthesized affinity ligand separation processes have now been fully accepted by the foremost regulatory authority, the USA's Food and Drug Administration, confirms a worldwide acceptance of the power of ligand design technologies.

See Colour Plate 1.

Further Reading

Briefs K-G and Kula M-R (1900) Fast protein chromatography on analytical and preparative scale using modified micro-porous membranes. *Chemical Engineering Science* 47: 141–149.

Burton SJ, Stead CV and Lowe CR (1988) Design and applications of biomimetic anthraquinone dyes. *Journal of Chromatography* 455: 201–216.

Chase HA (1994) Purification of proteins using expanded beds. *Trends in Biotechnology* 12: 296–305.

Dean PDG, Johnson WS and Middle FA (1985) *Affinity Chromatography: A Practical Approach*. Oxford: IRL Press.

Jones K (1990) A review of affinity chromatography. *Chromatographia* 32: 469–480.

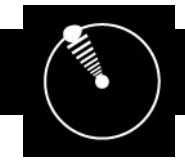
Kenny A and Fowell S (1990) *Methods in Molecular Biology: Practical Protein Chemistry*. New York: Humana Press.

Kopperschlager G (1994) Affinity extraction with dye-ligands. *Methods in Enzymology* 228: 121–129.

Walker JM and Gaastra W (1987) *Techniques in Molecular Biology*. London: Croom Helm.

DropBooks

CENTRIFUGATION



D. N. Taulbee and M. Mercedes Maroto-Valer, University of Kentucky-Center for Applied Energy Research, Lexington, KY, USA

Copyright © 2000 Academic Press

Introduction

Centrifugation is a mechanical process that utilizes an applied centrifugal force field to separate the components of a mixture according to density and/or particle size. The principles that govern particle behaviour during centrifugation are intuitively comprehensible. This may, in part, explain why centrifugation is seldom a part of post-secondary science curricula despite the broad range of scientific, medical and industrial applications in which this technique has been employed for well over 100 years. Applications that range from the mundane, industrial-scale dewatering of coal fines to the provision of an invaluable tool for biomedical research.

The first scientific studies conducted by Knight in 1806 reported the differences in orientation of roots and stems of seedlings when placed in a rotating wheel. However, it was not until some 60 years later that centrifuges were first used in industrial applications. The first *continuous* centrifuge, designed in 1878 by the Swedish inventor De Laval to separate cream from milk, opened the door to a broad range of industrial applications. About this same time, the first centrifuges containing small test tubes appeared. These were modest, hand-operated units that attained speeds up to 3000 rpm. The first electrically driven